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Novel Plant-specific Cyclin-dependent Kinase Inhibitors Induced by Biotic and Abiotic Stresses*

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The *EL2* gene of rice (*Oryza sativa*), previously classified as early response gene against the potent biotic elicitor *N*-acetylchitoheptaose and encoding a short polypeptide with unknown function, was identified as a novel cell cycle regulatory gene related to the recently reported *SIAMESE* (*SIM*) gene of *Arabidopsis thaliana*. Iterative two-hybrid screens, *in vitro* pull-down assays, and fluorescence resonance energy transfer analyses showed that *Orysa*;EL2 binds the cyclin-dependent kinase (CDK) CDKA1;1 and D-type cyclins. No interaction was observed with the plant-specific B-type CDKs. The amino acid motif ELERFL was identified to be essential for cyclin, but not for CDK binding. *Orysa*;EL2 impaired the ability of *Orysa*;CYCD5;3 to complement a budding yeast (*Saccharomyces cerevisiae*) triple *CLN* mutant, whereas recombinant protein inhibited CDK activity *in vitro*. Moreover, *Orysa*;EL2 was able to rescue the multicellular trichome phenotype of *sim* mutants of *Arabidopsis*, unequivocally demonstrating that *Orysa*;EL2 operates as a cell cycle inhibitor. *Orysa*;EL2 mRNA levels were induced by cold, drought, and propionic acid. Our data suggest that *Orysa*;EL2 encodes a new type of plant CDK inhibitor that links cell cycle progression with biotic and abiotic stress responses.

As in other eukaryotic organisms, in plants cell division is controlled by the timely and spatial activation of multisubunit complexes that are minimally composed of a catalytic cyclin-dependent kinase (CDK)⁴ and a regulatory cyclin subunit (1).

The model plant species *Arabidopsis thaliana* counts up to 12 CDKs and 49 cyclins that have been grouped into different types according to sequence similarity (2, 3). A-type CDKs are most closely related to the mammalian CDK1 and CDK2, contain the characteristic PSTAIRE amino acid sequence in their cyclin-binding domain, and play a role at both the G₁-to-S and G₂-to-M transition points. By contrast, the plant-specific B-type CDKs hold a divergent cyclin-binding domain and control the G₂-to-M transition only (1). The A- and B-type CDKs probably form complexes with cyclins of type A, B, and D. D-type cyclins respond to external and internal growth signals, such as hormones and carbohydrate levels (4, 5). They operate at the G₁-to-S transition point, although they might control the G₂-to-M transition as well (6–9). A-type cyclins are mainly produced from the onset of the S phase until the middle of the G₂ and B-type cyclins, specifically from G₂ until the end of mitosis (1, 10).

Because of their sessile life style, it is plausible that plants have developed mechanisms that allow them to adjust their cell cycle in response to environmental cues. Both biotic and abiotic stress stimuli negatively affect plant growth through the inhibition of the cell cycle machinery. Salinity inhibits *Arabidopsis* root growth by reducing the pool of dividing cells in the meristem (11, 12). Likewise, in leaves of wheat (*Triticum aestivum*) and maize (*Zea mays*), water stress induces a shortening of the meristem and prolongs cell cycle duration as a result of reduced CDK activity (13, 14). On the biotic side, cell cycle activity is inhibited in parsley (*Petroselinum crispum*) and tobacco (*Nicotiana tabacum*) cell cultures upon treatment with fungal elicitors (15, 16).

Perception of biotic and abiotic stress signals activates signaling cascades that trigger ion fluxes, kinase cascades, the generation of reactive oxygen species, and accumulation of antimicrobial hormones, such as abscisic acid (ABA) and jasmonic acid. These signaling molecules stimulate cell cycle checkpoints, resulting into an impaired G₁-to-S transition, slowing down of DNA replication, and/or delayed entry into mitosis (16–20). Still, insight is lacking into the molecular mechanisms that link the stress perception directly to the cell cycle machinery. Putative candidate proteins are CDK inhibitors (CKIs). In mammals, seven CKIs have been identified, which, based on structural and biochemical features, belong to two very distinct classes. Members of the INK4 family (p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, and p19^{INK4d}) are characterized by the presence of

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⁴ The abbreviations used are: CDK, cyclin-dependent kinase; ABA, abscisic acid; ADH, alcohol dehydrogenase; CKI, CDK inhibitor; FRET, fluorescence resonance energy transfer; GAL, galactose; GST, glutathione S-transferase; HB, homogenization buffer; ICK, interactor of Cdc2 kinase; KRP, Kip-related protein; SIM, SIAMESE; SMR, SIAMESE-related.

multiple ankyrin repeats and selectively inhibit G_1 -specific CDKs (CDK4 and CDK6). In contrast, inhibitors of the Kip/Cip family (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) bind and inhibit a broader range of CDKs involved in the control of the G_1 -to-S transition (21). Until recently, only one class of plant CKIs has been described whose members were designated as Kip-related proteins (KRPs), because of their similarity with the mammalian Kip/Cip proteins, but some members are also known as Interactors of Cdc2 Kinases (ICKs) (21). Of late, a second putative CKI was found, distantly related to the ICK/KRPs, known as SIAMESE (SIM) (22). SIM interacts with A-type CDKs and D-type cyclins, and its overproduction results into a strong inhibition of cell division activity. However, biochemical proof of its inhibitory activity is still lacking. No plant homologs to the mammalian INK4 or yeast inhibitors have been discerned so far (2).

Here, we demonstrate that the Orysa;EL2 protein of rice (*Oryza sativa*), recognized as a novel D-type cyclin-interacting protein, interferes with the ability of plant D-type cyclins to complement a yeast strain deficient in its G_1 cyclins, suggesting that Orysa;EL2 acts as an novel CKI. In agreement, Orysa;EL2 expression was able to rescue the multicellular trichome phenotype of *sim* mutants of *Arabidopsis*, whereas the recombinant Orysa;EL2 protein efficiently inhibited the activity of purified CDKs. The Orysa;EL2 gene was transcriptionally induced by biotic and abiotic stress treatments, implying that Orysa;EL2 might coordinate stress perception and cell cycle progression.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Experiments—The full-length Orysa;CYCD5;3 open-reading frame was amplified by PCR with gene-specific primers and subcloned into the pBD-GAL4 Cam vector (Stratagene, La Jolla, CA), resulting into the pBD-GAL4:CYCD5;3 bait plasmid. The Orysa;EL2 bait construct was obtained by cloning the Orysa;EL2 cDNA into a GATEWAY-modified pBD-GAL4 vector (Invitrogen, Carlsbad, CA). Orysa;EL2 cDNA was amplified from rice (*Oryza sativa* L. cv. Nipponbare) with gene-specific primers with *attB* adaptors, cloned into the pDONRTM201 ENTRY vector (Invitrogen) by an *attB* × *attP* (BP) recombination reaction, and subsequently mobilized into the pBD-GAL4 vector by an *attL* × *attR* (LR) recombination reaction. All constructs were confirmed by sequencing. The cDNA library used for the two-hybrid screens was prepared with RNA extracted from rice cell suspension cultures harvested 0, 3, 6, 9, and 12 days after subculture in fresh medium. Equimolar amounts (100 μ g) of total RNA from each sample were used to purify poly(A)⁺ mRNA with the Poly(A) Quick mRNA Isolation kit (Stratagene). The cDNA was synthesized and subcloned into the HybriZAP-2.1 λ vector according to the manufacturer's instructions (Stratagene). Approximately 2×10^6 independent plaque-forming units were produced, with an average insert size of 1 kb. The library was amplified once to yield 2×10^9 plaque-forming units/ml. Screens were performed with the PJ69-4a (MATa; 23) yeast strain according to a protocol described previously (23). The cDNA inserts from interacting clones were amplified by PCR and sequenced. For pairwise two-hybrid interactions, different combinations between bait (pBD-GAL4:OSCYCD5) and prey constructs were

transformed into yeast cells and assayed for their ability to grow on histidine-deficient minimal media at 30 °C.

Sequence Analysis—Conserved protein domains were detected with MEME (24), whereas domain matches on the different protein sequences were identified with a combination of MEME and HMMer (25). Multiple sequence alignments were generated with ClustalW (26).

Yeast Complementation—The yeast tetracycline-repressible expression vector pCM190 (27) was made GATEWAY compatible. The resulting pTHGW vector contained the *attR1* site of the GATEWAY cassette, directly upstream of the tetracycline-repressible iso-1-cytochrome C promoter. The rice cyclin Orysa;CYCD5;3 full-length coding sequence was transferred into the pTHGW destination vector from a pDONR-CYCD5;3 entry clone by LR cloning, yielding the pTH-CYCD5 vector. To obtain the pADH-EL2 expression vector, the GAL4 activation domain was removed from the pAD-GAL4 (Stratagene) by digestion, and the GATEWAY cassette C was inserted, resulting into the pADHGW construct. The Orysa;EL2-coding sequence was introduced into pADHGW by LR cloning. The resulting plasmids pTH-CYCD5 and pADH-EL2 (or pTHGW and pADHGW as controls) were transformed alone or in pairs into the yeast *CLN* mutant strain BF305-15d-21 (MATa *leu* 2–3, 112*his*3–11, 15*ura*3–52 *trp1 ade1 met14; arg5,6 GAL1-CLN3 HIS3::cln 1 TRP1::cln2*) with the Frozen-EZ Yeast Transformation II kit (ZymoResearch, Orange, CA). The complementation analyzed according to Hsieh *et al.* (28).

FRET Analysis—Onion epidermal cells were used for FRET analysis as described (22).

Sim Complementation Analysis—The full-length Orysa;EL2-coding region was introduced into the GATEWAY[®] compatible vector pLEELA-pGL2 (a gift from Dr. Arp Schnittger) by an LR recombination reaction, placing the Orysa;EL2 open-reading frame under direct control of the *GLABRA2* promoter. The resulting plasmid was introduced into *Agrobacterium tumefaciens* by electroporation, and subsequently into *sim-1* plants via the floral dip method (29). Transgenic plants were planted in soil, then sprayed with 1 mM phosphinothricin after the first leaves had established, and resistant plants were inspected for complementation of the *sim* phenotype.

Glutathione S-Transferase (GST) Pull-down Assays—The open-reading frames of Orysa;EL2 and Orysa;*el2*^{ELERLF} were cloned into pGEX4T-1 (GE-Healthcare, Little Chalfont, UK). The obtained plasmids were transformed in the *Escherichia coli* BL21-codonPlus(DE3)-RIL strain (Novagen, Madison, WI). *E. coli* cells were grown in a Luria-Broth medium to an A_{600} of 1 at 37 °C. Production of GST fusion proteins was induced by the addition of 1 mM isopropyl-D-galactoside for 6 h. The GST fusion proteins were purified with glutathione-Sepharose 4B (GE-Healthcare) according to the manufacturer's protocol. Subsequently, a total of 500 μ g of total protein extracted from a 2-day-old dividing *Arabidopsis* cell culture was mixed with 3 μ l of beads from a dilution series (100×, 10×, and 1×). The pull-down was performed in a total volume of 200 μ l of homogenization buffer (HB; 25 mM Tris-Cl, pH 7.6, 75 mM NaCl, 15 mM MgCl₂, 15 mM ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid, 15 mM *p*-nitrophenylphosphate, 60

mM β -glycerophosphate, 1 mM dithiothreitol, 0.1% Nonidet P-40, 0.1 mM Na_3VO_4 , 1 mM NaF, and protease inhibitor mixture P9599 (Sigma-Aldrich) and incubated on a rotating wheel for 2 h at 4 °C. The bead-bound fractions were washed three times with HB. The beads were divided in two parts: one part for kinase assay and one fraction was resuspended in 1 \times sodium dodecyl sulfate loading buffer and boiled. Proteins were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto Immobilon-P membranes (Millipore, Bedford, MA). Filters were blocked overnight at 4 °C in 3% (v/v) milk powder in 25 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20, and incubated for 1 h at 4 °C with a cdc2(anti-PSTAIR):sc-53 (1/1000) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-GST (1/2000) (GE-Healthcare) antibodies in blocking buffer. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated IgG diluted 1/10000 (GE-Healthcare) with a chemiluminescence system (Perkin-Elmer Life Sciences, Norwalk, CT).

Expression and Purification of *Orysa;EL2*—The *Orysa;EL2* gene was cloned in an expression vector for *E. coli* (30), containing a His-tagged NusA fusion partner. After induction at 20 °C with isopropyl- β -D-thiogalactopyranoside for 16 h, the cells were lysed and loaded on an IMAC column (Ni-Sepharose HP; GE-Healthcare). The fusion part was removed enzymatically with His-tagged caspase-3 at an engineered DEVD site immediately preceding the *Orysa;EL2* mature sequence. The protein was further purified on a mono-Q ion exchange column (GE-Healthcare) and a Ni-Sepharose IMAC column. The monomeric *Orysa;EL2* was polished on a gel filtration column while the buffer was changed to phosphate-buffered saline, concentrated, and stored at –80 °C. For kinase assays, *Arabidopsis* cells were harvested from actively dividing cell cultures and either used immediately or snap-frozen in liquid nitrogen and stored at –70 °C. The cells were ground in liquid nitrogen and proteins were extracted in HB. Equal amounts of total protein were incubated with 100 μ l of 25% (v/v) p10^{CKS1A}-Sepharose beads overnight at 4 °C on a rotary shaker. For immunoprecipitations, 300 μ g of total protein in HB were pre-cleared with 30 μ l of 50% (v/v) protein A-Sepharose beads (GE-Healthcare) for 1 h at 4 °C. After a short centrifugation, the pre-cleared supernatants were transferred to new Eppendorf tubes containing CDKA;1 (1/250) or CDKB1;1 (1/100) antibodies (31, 32) and incubated at 4 °C for 2 h. In the following step, 30 μ l of 50% (v/v) protein A-Sepharose was added, and the tubes were incubated on a rotating wheel for 1 h at 4 °C. Thereafter, beads were washed three times with 20 mM Tris-HCl, pH 7.4, 5 mM ethylenediaminetetraacetic acid, 2 mM ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 300 μ M phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin and pepstatin, and used for CDK activity reactions. Kinase assays were performed as described (33).

Stress Treatment, RNA Preparation, and Quantitative PCR—Seeds of rice were germinated in the dark in sand saturated with water. After 5 days, the germinating seedlings were transferred to 24-multiwell tissue culture plates (Falcon, BD Biosciences, San Jose, CA) filled with tap water, and grown at 55% relative humidity, a temperature regime of 28 °C day/22 °C night, with

an 11-h day length at 3,000 Lux light intensity for 2 more days. Seven-day-old seedlings at leaf stage 2 were exposed to heat (37 °C), cold (4 °C), drought, ABA (100 μ M), or propionic acid (1.5 mM). Control plants were kept in tap water. After stress treatment, the seedlings were cut at the crown region with a clean sterile blade. The seeds, roots, and first leaves were discarded. Each biological sample containing four seedlings was collected in triplicate. Total RNA and first-strand cDNA were prepared with the RNeasy plant mini kit (Qiagen, Hilden, Germany) and Superscript III first-strand cDNA synthesis kit (Invitrogen), respectively, according to the manufacturer's instructions. Multiplex PCR reactions were carried out on an LC480 Q-RT-PCR machine (Roche Diagnostics, Brussels, Belgium) in 384-well plates. Specific primers used were: 5'-CTCAGCAGCAGGGGAAGG-3' (forward primer) and 5'-AGGTGAAGGTTGGCATTATTGG-3' (reverse primer) for *Orysa;EL2* and 5'-TTGTGTTGGACTCTGGT-GATGG-3' (forward primer) and 5'-CCGTCAGGATCTTCATGAGGTAAT-3' (reverse primer) for *Orysa;ACTIN1* (Os03g50890). The transcript level of the *Orysa;ACTIN1* gene was used as an internal control. cDNA was amplified for multiplex PCR with SYBR Green I master mix (Roche Diagnostics) according to the manufacturer's instructions. Relative expression levels of *Orysa;EL2* were determined by the comparative Δ Ct method (34) and normalized by subtracting the average Ct values of the triplicate reactions for the *Orysa;ACTIN1* gene under control conditions from those of the *Orysa;EL2*, resulting into the Δ Ct values. The Δ Ct values of the treated samples were subtracted from the Δ Ct values of the control samples, resulting into Δ Ct values. The fold changes of the gene expression between two samples were calculated with the Δ Ct in the formula: fold change = $2^{\Delta\Delta\text{Ct}}$.

Meta-analysis of *Arabidopsis* *EL2*-like Gene Expression—With the "Response Viewer" tool of GENEVESTIGATOR, the expression profiles of genes to different stimuli were analyzed (35). Only biotic and abiotic stress treatments with a more than 2-fold change in transcription level for at least one of the *EL2*-like genes were taken into account. Fold-change values were hierarchically clustered for genes and experiments by average linkage in "Multiple Experiment Viewer" of The Institute for Genome Research.

RESULTS

***Orysa;EL2* Is a Novel D-type Cyclin-interacting Protein**—To identify novel rice proteins that control the G₁-to-S transition of the plant cell cycle, a yeast two-hybrid screen was performed with a D-type cyclin of rice *Orysa;CYCD5;3* (Os03g10650) as bait. For the screening, a galactose 4 (GAL4) activation domain cDNA fusion library was used, constructed with RNA isolated from an actively dividing rice cell suspension culture. A total of 10⁶ independent yeast cotransformants were screened, resulting into 31 positive colonies. Most cDNA clones (77%) encoded a small protein of 106 amino acids (predicted molecular mass of 11.6 kDa), identical to the *Orysa;EL2* protein (Os03g01740) whose gene had originally been identified as an early-response gene upon elicitation with *N*-acetylchitoheptaose, a potent biotic elicitor for phytoalexin biosynthesis genes (36). *Orysa;EL2* has no sequence homology with any characterized func-

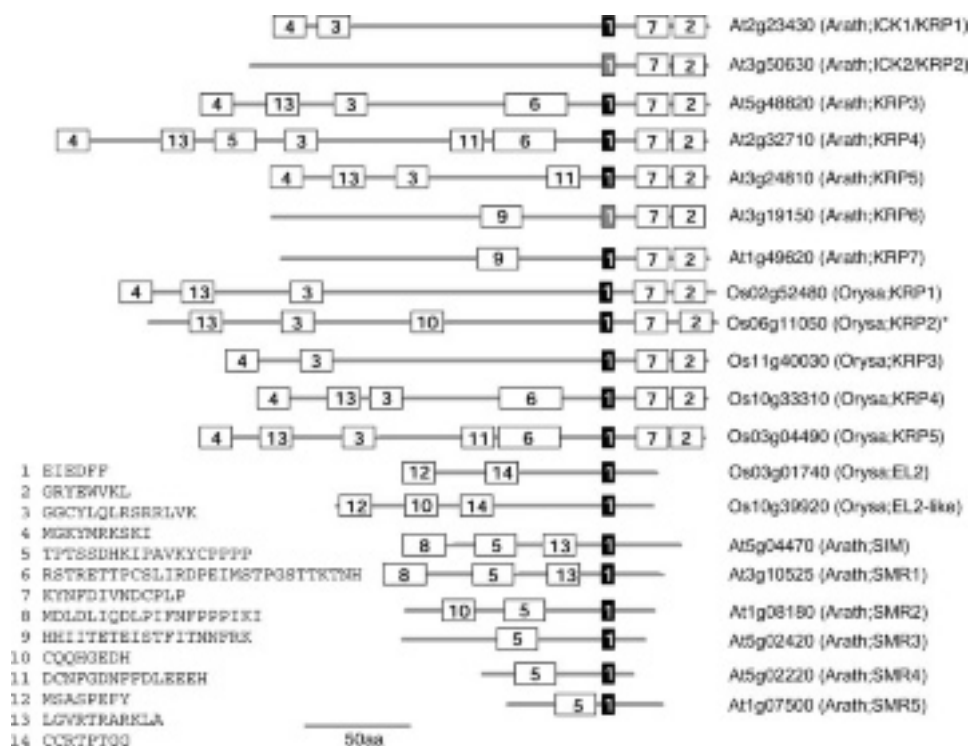


FIGURE 1. Schematic overview of the domain organization in ICK/KRP and EL2-like proteins of rice and *Arabidopsis*. Motif 1 conserved between all proteins is marked in black. Gray boxes are domains identified by HMMer but not by MEME (see "Experimental Procedures"). aa, amino acids.

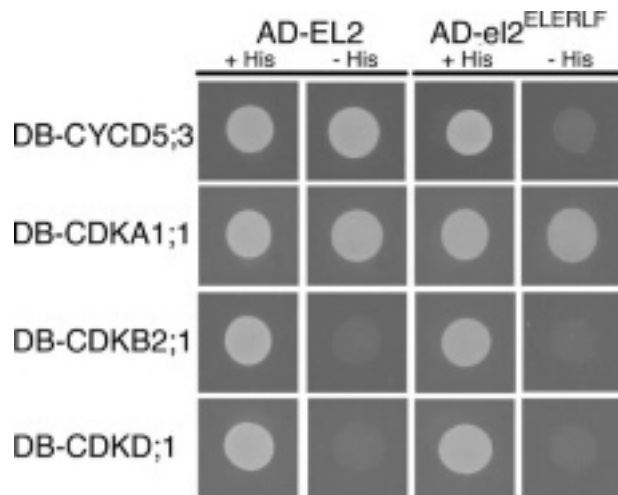


FIGURE 2. The ELERLF domain required for cyclin but not CDK binding. Yeast PJ69-4a cells were transformed with a plasmid encoding a GAL4 DNA-binding domain fused with either Orysa;CYCD5;3, Orysa;CDKA1;1, Orysa;CDKB2;1, or Orysa;CDKD;1 (DB-CYCD5;3, DB-CDKA1;1, DB-CDKB2;1, or DB-CDKD;1), together with the AD-EL2 or AD-el2^{ELERLF} plasmids, encoding a fusion protein between the GAL4 activation and the wild-type (AD-EL2) or mutant (AD-el2^{ELERLF}) Orysa;EL2 protein, respectively. Transformants were streaked on plates with (+His) or without (-His) histidine. Reconstitution of GAL4 activity restored the ability to grow on His-deficient medium.

tional protein but shares a small peptide domain of six amino acids with the ICK/KRPs (Fig. 1). Alignment of Orysa;EL2 with ICK/KRPs yielded the E[ILM][ED][EDR][FL]F consensus motif (in bold, the most frequently occurring residues). Sequence similarity searches with this domain as a query identified one related protein from rice (Fig. 1). Also six *Arabidopsis* proteins were found, among which the recently described SIM

protein (22). The EIEDFF domain resides into the mapped cyclin-binding domain of KRPs (37). To test whether the ELERLF motif is required for cyclin binding, it was mutated into a stretch of alanine residues, resulting into the mutant el2^{ELERLF} protein that did not interact with Orysa;CYCD5;3 (Fig. 2).

Orysa;EL2 Binds Orysa;CDKA1;1—To identify other Orysa;EL2-interacting proteins, a two-hybrid screen was performed with the Orysa;EL2 protein as bait. Among the interacting clones, Orysa;CYCD4;1 (Os09g29100) and CDKA1;1 (Os03g02680) were identified. The interaction between Orysa;EL2 and Orysa;CDKA1;1 did not depend on the ELERLF motif (Fig. 2). Orysa;EL2 did not associate with other CDKs tested, such as Orysa;CDKB2;1 and Orysa;CDKD;1 (Fig. 2).

The protein-protein interactions observed with the two-hybrid system were confirmed by FRET experiments. As a positive-control FRET protein pair, the *Arabidopsis* transcription factor TGA (At5g06960), whose self-interaction in plants had previously been detected by FRET analysis (38), was used, and as a negative control, the noninteracting LexA-nuclear localization signal (NLS) and TGA5 proteins (39). FRET efficiency was evaluated by the increase in donor fluorescence after photobleaching of the acceptor molecule, in cells that produced YFP:CYCD5;3 (as acceptor) together with either CFP:EL2 or CFP:el2^{ELERLF} (as donors). When measuring the fluorescence intensity of CFP:EL2 before and after YFP photobleaching, an average increase in fluorescence of 11.4% was seen for CFP:EL2, demonstrating that Orysa;EL2 interacted *in vivo* with Orysa;CYCD5;3 (Fig. 3; Table 1). No FRET was observed for CFP:el2^{ELERLF}. By contrast, an efficient FRET signal was noticed for both CFP:EL2 and CFP:el2^{ELERLF} in the presence of YFP:CDKA1;1 (Table 1), again indicating that the interaction between Orysa;EL2 and Orysa;CDKA1;1 is independent of the ELERLF motif. No significant association was observed between Orysa;EL2 and Orysa;CDKB2;1 or Orysa;CDKD;1. Interestingly, both Orysa;EL2 and Orysa;CYCD4;1 localized specifically into the nucleus (Fig. 3).

To confirm the interaction of Orysa;EL2 with CDKA1;1, an *in vitro* pull-down experiment was done with a GST-EL2 fusion protein. In addition, a GST protein fused to the Orysa;el2^{ELERLF} protein with the mutated cyclin-binding domain was used. After extensive washing, bound proteins were eluted and used for protein gel blot analyses with an anti-PSTAIRE antibody, which specifically recognizes A-type CDKs. Whereas no CDKs were found to associate with the mock-treated sample, a clear CDK signal was seen for the GST-EL2 beads (Fig. 4A). Also the

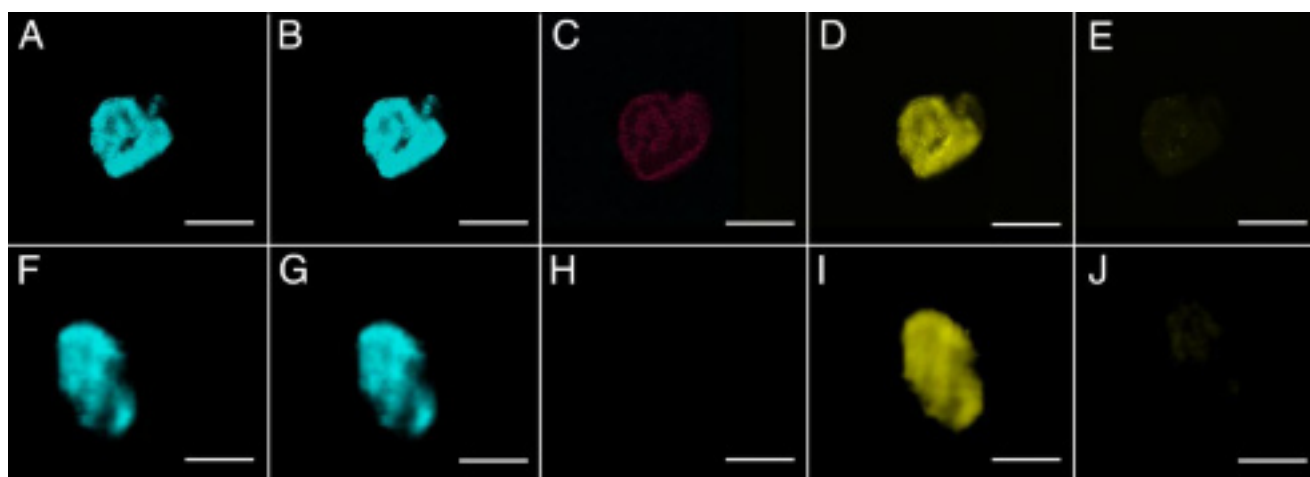


FIGURE 3. *In vivo* binding between Orysa;EL2 and Orysa;CYCD5;3. A and B, CFP:EL2 before and after YFP bleaching respectively. C, pre-bleached CFP:EL2 signal subtracted from the post-bleached CFP:EL2 signal represented in false color showing 12% increase in CFP fluorescence intensity, indicating interaction between CFP:EL2 and YFP:CYCD5;3. D and E, YFP:CYCD5;3 before and after YFP bleaching, respectively. F and G, CFP:el2^{ELERLF} before and after YFP bleaching, respectively. H, pre-bleached CFP:el2^{ELERLF} signal subtracted from the post-bleached CFP:el2^{ELERLF} signal, without increase in CFP fluorescence intensity, indicating no interaction between CFP:el2^{ELERLF} and YFP:CYCD5;3. I and J, YFP:CYCD5;3 before and after YFP bleaching, respectively. Scale bars, 2 μ m.

TABLE 1
Orysa;EL2 protein-protein interactions determined by FRET

Donor (CFP fusion)	Acceptor (YFP fusion)	FRET Efficiency(%) ^a	n ^b	p ^c	Interaction
TGA5	TGA5	13.1	10	<0.0001	Y
TGA5	LexA-NLS	0.1	10	1.00	N
EL2	CYCD5;3	11.4	10	<0.0001	Y
el2 ^{ELERLF}	CYCD5;3	0.1	10	0.99	N
EL2	CDKA1;1	12.0	10	<0.0001	Y
el2 ^{ELERLF}	CDKA1;1	8.5	10	<0.0001	Y
EL2	CDKB2;1	0.4	10	0.69	N
EL2	CDKD;1	0.8	10	0.19	N

^a The Acceptor Photobleaching method was used to determine the FRET efficiency (see "Experimental Procedures").

^b Number of nuclei analyzed.

^c Student's *t* tests were performed for each data set. *p* indicates the statistical significant difference between each data set and the negative control values.

mutant Orysa;el2^{ELERLF} associated with CDKs, albeit with reduced affinity. A dilution series of bead-bound recombinant proteins was prepared to obtain pull-downs with similar amounts of CDKs. The CDK activity associated with the purified complexes was measured. More kinase activity was detected with the GST-el2 than the GST-EL2 fraction (Fig. 4B), indicating that the ability of Orysa;EL2 to interact with cyclins is essential to exert its role as a CDK inhibitor.

Orysa;EL2 Abolishes Rescue of the CLN Budding Yeast Mutant by Plant D-type Cyclins—A functional interaction between Orysa;EL2 and Orysa;CYCD5;3 was demonstrated with the mutant budding yeast strain BF305-15d-21 that lacks two out of its three G₁-specific cyclins (*CLN1* and *CLN2*) and is conditionally deficient for the third one (*CLN3*). In the presence of galactose, the *CLN3* gene is expressed and cells are able to divide. When transferred to glucose-containing media, *CLN3* expression is repressed, and cells become arrested in G₁ (40). When BF305-15d-21 cells were transformed with an expression vector containing the Orysa;CYCD5;3 gene (pTH-CYCD5), cells resumed growth (Fig. 5), indicating that Orysa;CYCD5;3 encodes a functional cyclin able to complement a *CLN* mutant yeast strain. By contrast, when the Orysa;CYCD5;3-expressing yeast strain was transformed with an Orysa;EL2-expressing construct, containing the Orysa;EL2 cDNA

sequence under the control of the alcohol dehydrogenase 1 (*ADH1*) promoter, Orysa;EL2 hampered the ability of Orysa;CYCD5;3 to complement the BF305-15d-21 strain (Fig. 5). Cell proliferation was completely inhibited, illustrating that Orysa;EL2 blocked the function of Orysa;CYCD5;3 in yeast.

Orysa;EL2 Is a Functional CKI in Vitro—The observed interaction of Orysa;EL2 with CDKs and cyclins and its interference with the capability of the Orysa;CYCD5;3 cyclin to complement the yeast *CLN* mutant suggested that Orysa;EL2 could operate as an inhibitor of CDK-cyclin complexes. To test this hypothesis, CDK-cyclin complexes were purified from cell suspension culture extracts either by affinity chromatography with p10^{CKS1A}-Sepharose beads or by immunoprecipitation with specific antibodies for CDKA1;1 and CDKB1;1. Recombinant Orysa;EL2 was added to the affinity-purified CDKs and kinase activity was measured with histone H1 as substrate. CDK activity did not decrease significantly upon addition of Orysa;EL2 to p10^{CKS1A}-Sepharose-purified complexes (Fig. 4, C and D). By contrast, Orysa;EL2 was found to be a potent inhibitor of immunopurified CDKA1;1 complexes (Fig. 4, C and D). CDKA1;1 activity was only partially inhibited. Addition of a higher quantity of Orysa;EL2 did not result in a stronger inhibition of CDK activity (data not shown). Orysa;EL2 inhibited only slightly B-type CDK activity at the highest dose applied (Fig. 4, C and D).

Orysa;EL2 Complements the Arabidopsis Sim Phenotype—Orysa;EL2 shows sequence similarity to the recently reported *SIM* gene (22). In *Arabidopsis*, trichome cells are unicellular. By contrast, recessive mutations in the *SIM* gene gives rise to multicellular trichomes, indicative that *SIM* is required to suppress cell division during hair development, according to its anticipated role as CKI. To test whether Orysa;EL2 was able to complement the *sim* trichome phenotype, *sim* mutant plants were transformed with a construct harboring the Orysa;EL2 construct under the control of the trichome-specific *GLABRA2* promoter. In 12 out of the 14 transgenic lines obtained, Orysa;EL2 was able to completely rescue the multicellular trichome

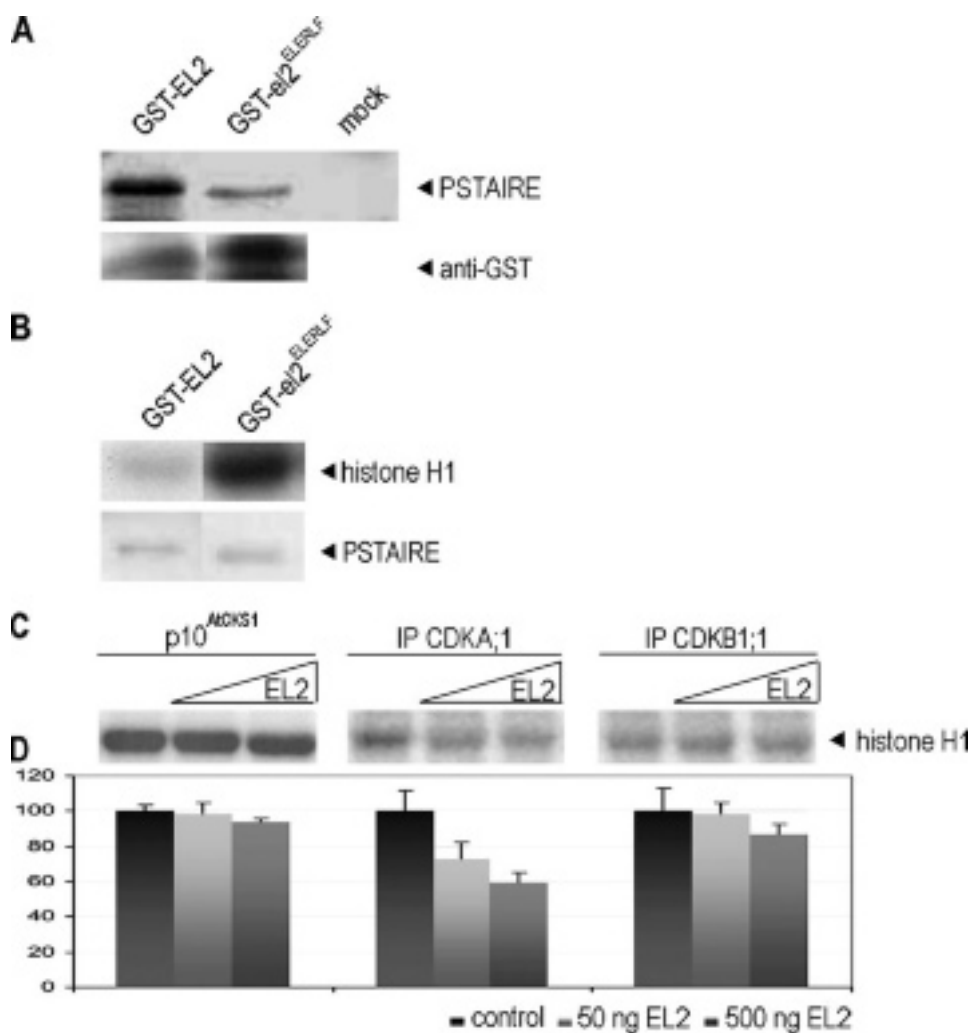


FIGURE 4. *In vitro* binding and inhibition of A-type CDKs by Orysa;EL2. *A*, total plant extracts incubated with GST-EL2, mutated GST-el2^{ELERLF}, or mock-treated. Associated proteins were pulled-down with glutathione-Sepharose beads. Bound proteins were used for a protein gel blot with a PSTAIRE-specific antibody. A protein gel blot with an anti-GST antibody was used to measure the amount of pulled-down GST-EL2 or GST-el2^{ELERLF}. *B*, activity measurement of CDKs associated with GST-EL2 and GST-el2^{ELERLF} histone H1 as a substrate. *C*, CDKs purified from protein extracts with either p10^{CKS1A}-Sepharose beads or specific anti-CDKA;1 and anti-CDKB1;1 antibodies. Pulled-down kinase activity was measured in increasing levels of recombinant Orysa;EL2 protein with histone H1 as substrate. *D*, relative quantification of two independent kinase activity measurements as depicted in *C*. Kinase activity in the absence of Orysa;EL2 arbitrarily set at 100%. Bars indicate S.E.

phenotype (Fig. 6), demonstrating that Orysa;EL2 and Arath;SIM are functionally related.

Orysa;EL2 Is Transcriptionally Induced upon Stress Treatment—Because of its observed elicitation with *N*-acetylchitoheptaose, the transcriptional response of Orysa;EL2 toward different stress treatments was tested in 7-day-old rice seedlings. Stresses applied included heat (37 °C), cold (4 °C), and drought. Steady-state mRNA levels were slightly reduced by heat, but remarkably strongly induced by 4 h of cold treatment (Fig. 7*A*). This induction was even more pronounced after 24 h of cold (Fig. 7*B*). At this time point, Orysa;EL2 was also induced by drought. Transcript levels did not change upon treatment with the antimitogenic hormone ABA (Fig. 7, *A* and *B*), suggesting that induction of Orysa;EL2 by cold and drought occurred in an ABA-independent manner. Orysa;EL2 was also up-regulated by propionic acid that triggers cytoplasmic acidifi-

cation, a downstream effect in *N*-acetylchitoheptaose elicitation (41).

With the GENEVESTIGATOR toolbox, the expression pattern of the six *Arabidopsis* SIM/EL2-like genes in response to different biotic and abiotic stress treatments was examined in more than 2,500 publicly available microarray experiments (35). The different family members were induced under various stress conditions, albeit with different specificity (Fig. 7*C*). Arath;SMR5 (*At1g07500*) and Arath;SMR3 (*At5g02420*) were activated by all or most stress conditions, respectively, whereas other EL2-like genes responded more specifically. Interestingly, distinct family members reacted often in a complementary manner; for example, Arath;SIM (*At5g04470*) and Arath;SMR1 (*At3g10525*) were induced by the nematode *Heterodera schachtii*, but down-regulated upon infection with the pathogen *Pseudomonas syringae*, whereas Arath;SMR2 (*At1g08180*) and Arath;SMR3 (*At5g02420*) had the opposite behavior.

DISCUSSION

We identified the Orysa;EL2 protein as a potent key regulator in the series of events that might transduce the early response of biotic and abiotic stresses directly to the cell cycle. Orysa;EL2, previously characterized as a gene induced within minutes after addition of the elicitor *N*-acetylchitoheptaose or purified flagellin protein of the pathogen *P. avenae* (36, 42), was demonstrated here to encode a D-type cyclin-binding protein that inhibits CDK activity. EL2-like proteins can be found in mono- and dicotyledonous plants, but no homologs are known in non-plant species, indicating that this family of CKIs is plant-specific.

Recombinant Orysa;EL2 protein specifically inhibited CDKA;1, but not CDKB1;1 activity, a feature shared with the previously described ICK/KRPs (43). The interaction with CDKA;1 must probably be direct, because both the wild-type Orysa;EL2 and its mutant isoform with deleted cyclin-binding domain pulled-down the CDK subunit. However, compared with the wild-type isoform, the mutant Orysa;el2 was less efficient in pulling down CDKs, indicating that, besides a direct interaction, Orysa;EL2 also partially interacts indirectly with CDKs, probably through the binding of cyclins. Remarkably,

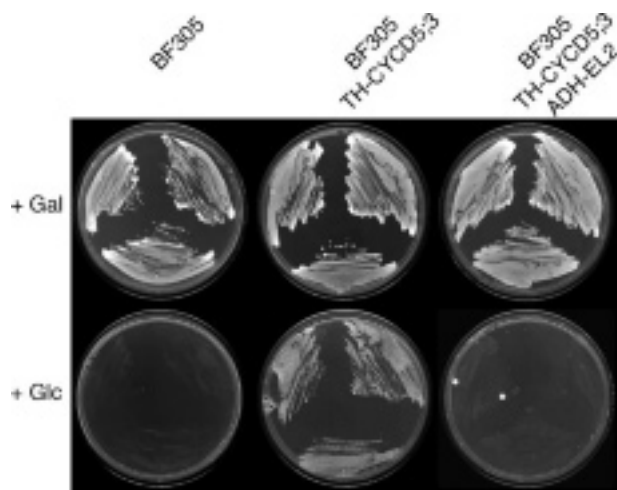


FIGURE 5. *Oryza*;EL2 interference with the ability of plant D-type cyclins to complement yeast *CLN* mutants. BF305-15d-21 cells, conditionally deficient for *CLN* cyclins, were transformed with only the *Oryza*;CYCD5;3-expressing construct (TH-CYC5;3), or in combination with an *Oryza*;EL2-expressing plasmid (TH-CYC5;3 + ADH-EL2). Cells were grown under conditions where the *CLN3* cyclin gene was expressed (on galactose-containing medium; +Gal) or repressed (on glucose-containing medium; +Glc). For each experiment, three independent colonies were tested.

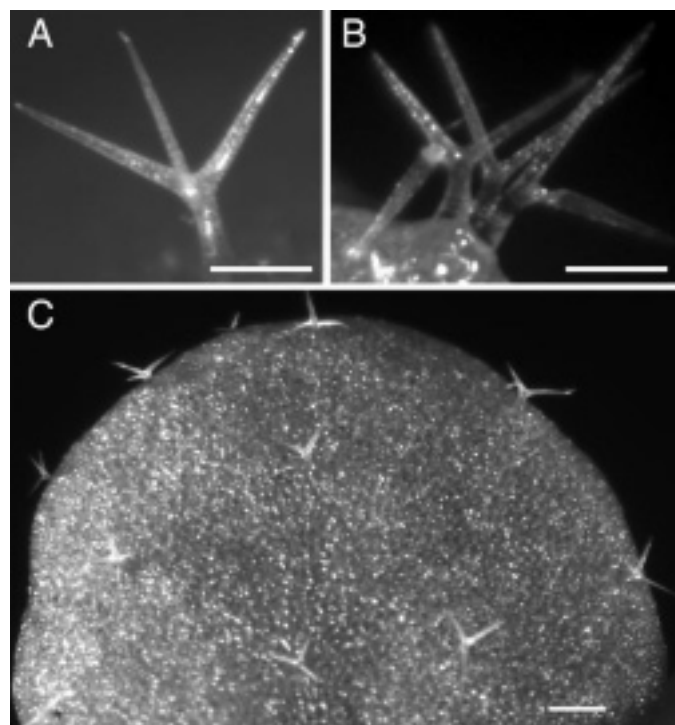


FIGURE 6. *Sim* complementation by *Oryza*;EL2. A, wild-type trichome. B, *sim* trichome. C, complementation of *sim* loss-of-function phenotype by pGL2:EL2. Bars, 100 μ m.

CDK complexes purified on p10^{CKS1At} beads were not inhibited, suggesting that either CKS1At and *Oryza*;EL2 compete for the same CDK-binding site or that p10^{CKS1At} lacks affinity for the *Oryza*;EL2 target complexes. Human p9^{CKShs1} and yeast p13^{SUC1} do not bind the mammalian G₁-specific CDK4 and CDK6 complexes (44, 45). Analogously, fission yeast (*Schizosaccharomyces pombe*) cdc2 associates only to p13^{SUC1} during mitosis (46). Selective CDK binding by p13^{SUC1} has been demonstrated for maize as well, where only M- but not S-associated

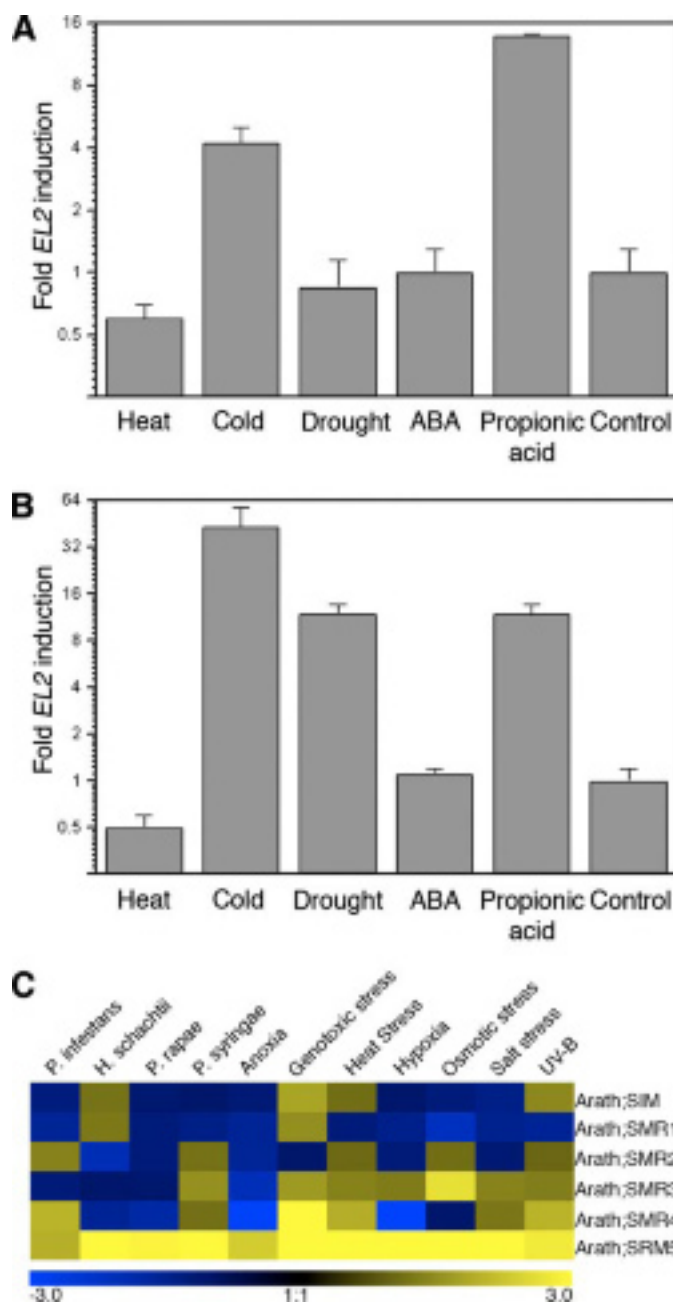


FIGURE 7. Stress induction of *Oryza*;EL2 and *Arabidopsis* EL2-like genes. A and B, seven-day-old rice seedlings subjected to the indicated stresses for 4 h and 24 h, respectively. *Oryza*;EL2 transcript levels were measured by real-time quantitative PCR. Bars indicate S.E. ($n = 3$). C, hierarchical average linkage clustering of *Arabidopsis* EL2-like genes in publicly available microarray experiments. Data were obtained with GENEVESTIGATOR, a data base and Web browser data-mining interface for Affymetrix GeneChip data (Santa Clara, CA). Code: blue, down-regulation; yellow, up-regulation; black, no change.

CDK complexes were found to bind p13^{SUC1} (47). Therefore, the lack of inhibition of p10^{CKS1At}-associated CDKs complexes by *Oryza*;EL2 might indicate that the main targets of *Oryza*;EL2 can be found among G₁/S-phase-associated CDK complexes. In support for a role during G₁-to-S, *Oryza*;EL2 interacts only with D-type cyclins and not with A- or B-type cyclins. Analogously as for mammals, a model has been put forward for plants in which D-type cyclins act as primary sensors linking external and internal growth signals with cell division activity, as illus-

trated by the cytokinin-independent growth of *Orysa*;CYCD3;1-overexpressing calli or the delayed activation of the cell cycle machinery in seeds of D-type cyclin knock-out lines when sown under favorable conditions (48, 49). The specific interaction of *Orysa*;EL2 with D-type cyclins suggests that the signaling cascades that arrest the cell cycle in response to biotic and abiotic stress signals directly impinge on the CYCD/CDK complexes. In such a model, the balance between CYCD/CDK complexes and EL2-like proteins might be a critical parameter to decide whether cells divide or not.

In mammals, interaction between Kip/Cip proteins and cyclins is controlled by the presence in the CKIs of one or more ZRXL amino acid motifs (with Z basic or cysteine and X preferentially basic). This ZRXL motif is found in other cyclin-interacting proteins, such as E2F1, p107, and p130 (50, 51) and is present in most *Orysa*;EL2-related sequences, but only in some of the ICK/KRPs. Moreover, mutation of the ZRXL motif does not impair the binding of ICK2/KRP2 with D-type cyclins,⁵ suggesting that a motif different from ZRXL controls the interaction of ICK/KRPs and EL2-like proteins with D-type cyclins. By mutational analysis, we identified the EIEDFF domain that is shared by all EL2-like proteins and ICK/KRPs as a cyclin-binding motif. This particular domain resides into the presumed cyclin-binding domain of ICK/KRPs (37). Moreover, its mutation hampers the association between *Orysa*;EL2 and *Orysa*;CYCD5;3 and is probably important for CDK inhibition. The EIEDFF motif can be found also in other potential plant cyclin-interacting proteins, such as retinoblastoma-binding protein 2-like proteins and the DNA mismatch repair protein PMS2. EL2-like and ICK/KRPs share some other small peptide sequence proteins, but, because they are only shared between a few members of each protein class, they might play a role in fine-tuning the activity of specific family members.

Orysa;EL2 was able to complement the *sim* trichome phenotype, illustrating that both genes are functionally related. Interestingly, in *sim* mutants, the trichomes do not only undergo mitotic cell division, but display as well an inhibition of their endoreduplication program (52). These data point toward a role for the SIM protein at the mitosis-to-endocycle switch during trichome development. Our results on *Orysa*;EL2 suggest that this transition might be achieved through inhibition of specific CDK/cyclin complexes. Therefore, the *EL2*/*SIM* genes might not only operate as sensors of antimitogenic stimuli, but also play important roles during developmental processes.

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⁵ L. De Veylder, unpublished results.

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